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Dysfunctional neutrophil effector organelle mobilisation and microbicidal protein release in alcohol-related cirrhosis

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Abbreviations: Alcohol-related cirrhosis: ALD cirrhosis; Multi-organ failure: MOF; Healthy Control: HC, Child Pugh score: CP, Model for end stage liver disease score: MELD.

New and Noteworthy: Neutrophil granule release is dysregulated in patients with alcohol-related cirrhosis with augmented effector organelle mobilisation and microbiocidal protein release.

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Abstract

Background and Aims: Patients with alcohol-related cirrhosis (ALD) are prone to infection. Circulating neutrophils in ALD are dysfunctional and predict development of sepsis, organ dysfunction and survival. Neutrophil granules are important effector organelles containing a toxic array of microbicidal proteins, whose controlled release is required to kill micro-organisms whilst minimising inflammation and damage to host tissue. We investigated the role of these granular responses in contributing to immune disarray in ALD.

Methods: Neutrophil granular content and mobilisation was measured by flow cytometric quantitation of cell-surface/intracellular markers, [secretory vesicles (CD11b), secondary granules (CD66b) and primary granules (CD63; myeloperoxidase)] before and after bacterial stimulation in 29 patients with ALD cirrhosis (15 abstinent; 14 actively drinking) compared to healthy controls (HC). ImageStream Flow Cytometry characterised localisation of granule subsets within the intracellular and cell-surface compartments. The plasma cytokine environment was analysed using ELISA/Cytokine Bead Array.

Results: Circulating neutrophils were primed in the resting state with upregulated surface expression of CD11b ($p=0.0001$) in a cytokine milieu rich in IL-8 ($p<0.001$) and lactoferrin ($p=0.035$). Neutrophils showed exaggerated mobilisation to the cell surface of primary granules at baseline ($p=0.001$) and in response to fMLP ($p=0.009$) and *E. coli* ($p=0.0003$). There was no deficit in granule content or mobilisation to the cell membrane in any granule subset observed. Paradoxically, active alcohol consumption abrogated the hyper-responsive neutrophil granular responses compared to their abstinent counterparts.

Conclusions: Neutrophils are pre-primed at baseline with augmented effector organelle mobilisation in response to bacterial stimulation; neutrophil degranulation is not a mechanism leading to innate immunoparesis in ALD.

New and Noteworthy:

- Neutrophil granules are upregulated in alcohol-related cirrhosis (ALD) at baseline and demonstrate augmented responses to bacterial challenge.
- The granular responses in ALD did not contribute to the observed functional deficit in innate immunity, but rather, were dysregulated and hyper-responsive which may induce bystander damage to host tissue.
- Paradoxically, active alcohol consumption abrogated the excessive neutrophil granular responses to bacterial stimulus compared to their abstinent counterparts.

Background

The incidence and prevalence of cirrhosis is fast increasing and it is now the fifth most common cause of death in the United Kingdom with mortality rates predicted to double within 20 years (10). Susceptibility to infection remains one of the main concerns in patients with cirrhosis with a variety of components of the innate immune system reported to be dysfunctional. Bacterial infection is demonstrable in 34-44% of patients admitted to hospital (8, 18) and the risk of infection correlates with the severity of cirrhosis (11). Infections frequently precipitate decompensation and multiple organ failure (MOF) and are associated with a high mortality rate; infections increase mortality more than three-fold in cirrhosis and confer a damning prognosis, with 30% of patients dying in the first month after infection and 60% after 1 year (5, 17).

Neutrophils are key players in the innate immune system providing the first line of defence against bacterial infection and constitute 40-60% of the white blood cell population. Neutrophils travel in a resting state in the healthy adult circulation with their microbicidal proteins stored intracellularly in granules and with low levels of reactive oxygen species (ROS) generated in the resting state (7). A number of abnormalities in neutrophil physiology have been reported in the context of cirrhosis; neutrophils have been shown to have impaired chemotaxis (12, 15, 20), opsonisation (26, 35), phagocytosis (19, 30) and elevated spontaneous ROS production (27). Furthermore, the degree of neutrophil dysfunction observed in patients with cirrhosis has been shown to be predictive of the risk of infection, organ failure and mortality (34). Neutrophil dysfunction in cirrhosis has been shown to be reversible with *ex-vivo* removal of endotoxin from patient plasma reducing oxidative burst and increasing phagocytic function (25) and normalisation of neutrophil cytokine profiles in alcoholic hepatitis after steroid administration (33).

Neutrophil granules are a diverse population of intracellular organelles that develop at sequential stages of myeloid differentiation and contain a specific milieu of proteins (representative of the evolving cellular transcriptome) with targeted functions. Neutrophil granules are mobilised and released at the cell surface and in the endo-phagosome in ordered sequence throughout neutrophil activation (6). Granule release is necessary for adhesion, diapedesis, chemotaxis and the eventual release of potent microbicidal peptides

at sites of inflammation and infection. This ordered and controlled sequence serves to minimise bystander damage to host tissue in healthy states (31) [Figure 1].

Figure 1

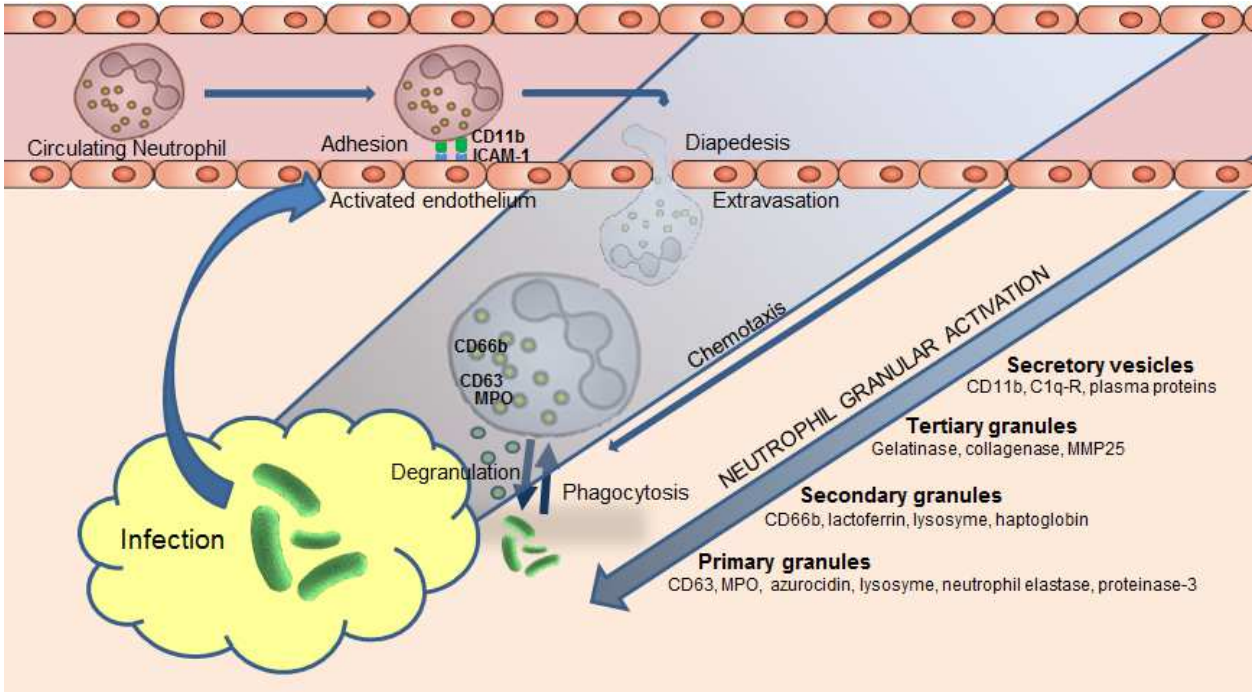


Figure 1: Graphic representation of neutrophil function, demonstrating conversion from a circulating inactive immune cell to targeted degranulation and phagocytosis at sites of inflammation (modified from Borregaard et. al (7)). The process occurs via sequential control of neutrophil granules which contain specific arrays of proteins which serve specific and timely functions relating to neutrophil activation.

Initial interaction occurs with the vascular endothelium via selectins and cell adhesion molecules which facilitate rolling, adhesion and subsequently diapedesis across the endothelial membrane into the interstitial space. This in turn allows hierarchical activation of the neutrophil with mobilisation of granules to the cell surface and release of chemokines, cytokines and microbicidal proteins in a time ordered sequence, allowing the neutrophil to migrate to areas of inflammation and release microbicidal proteins and reactive oxygen species at targeted areas.

Secretory vesicles are formed late in neutrophil maturation via endocytosis and contain membrane-bound molecules including CD11b (32) that aid in neutrophil adhesion and migration; these organelles are the easiest granule sub-type to mobilise to the cell membrane. Tertiary (gelatinase) granules contain an array of metalloproteinases which are felt to aid neutrophil migration within the interstitial tissue but are not interrogated in this study. Secondary (specific) granules are characterised by the presence of lactoferrin (14) and other antimicrobial proteins and have CD66b as a granule-marker (16). Secondary granules are released at sites of infection alongside primary granules. CD63 is a lysosomal membrane protein located on neutrophil primary (azurophil) granules (13) and mobilises to the cell membrane on stimulation where primary granules release their microbicidal contents which include myeloperoxidase (MPO) (28).

Data so far on the role of neutrophil granules in innate immune function in the context of cirrhosis is sparse and contradictory with studies demonstrating both normal degranulation patterns and impaired granule release upon response to bacterial stimulation (22, 30). Neutrophils have been shown to have reduced MPO release in response to bacterial peptide stimulation in the context of ALD in addition to reduced cell surface CD11b expression (9, 24). Further characterisation of this immunological deficit, crucial to innate immune system function, will enhance understanding of one of the major causes of morbidity and mortality in liver disease and potentially elucidate therapeutic targets.

We hypothesised that either a quantitative deficit in neutrophil granular content exists, or that there is an impairment of response to bacterial stimulation in terms of mobilisation of granule content and extracellular release; these contributing to the observed increased risk of bacterial infection in cirrhosis. The aim of this study was to characterise neutrophil granule mobilisation and release in patients with alcohol-related cirrhosis (ALD cirrhosis) compared to healthy controls (HC) in order to further delineate mechanisms of innate immune system compromise in ALD patients and identify potential therapeutic targets for intervention.

Materials and Methods

Study Design

Twenty nine patients with ALD cirrhosis were recruited including 15 actively drinking and 14 abstinent and compared to HC (n=12). The patients with ALD cirrhosis were recruited from both outpatient clinics and inpatient wards. A history of excess alcohol intake was defined from thorough clinical history as >80 g/day for men and >60 g/day for women. Abstinence was defined as >6 months without alcohol consumption. ALD cirrhosis was defined on the presence of either histological criteria, characteristic radiological findings or typical clinical presentation encompassing the presence of ascites, varices or encephalopathy. Alcoholic hepatitis was defined clinically with a typical history of excess alcohol intake, biochemical parameters meeting a minimum modified Maddrey's discriminant function of ≥32 (23) and the absence of other causes of liver disease. Patients were followed longitudinally for 1 year and data on subsequent development of sepsis, death or liver transplantation were recorded.

Inclusion Criteria

Patients with ALD were included if they were >18 and <75 years and had provided consent for study inclusion. Healthy age- and sex-matched, non-smoking volunteers with no history of liver disease were used as HC. The HC alcohol intake was <20g/day and volunteers had not drunk alcohol or exercised excessively in the 72 hours prior to blood being drawn.

Exclusion Criteria

Patients were excluded from the study if, on presentation, they had evidence of bacterial, viral or fungal infection on the basis of clinical examination, laboratory and radiological investigation, malignancy, and any coexisting history of immunodeficiency, HIV infection or glycogen storage disease. Patients were also excluded if they were taking any concurrent immunosuppressive medications.

Consent and Data Collection

The study was performed in accordance with the Declaration of Helsinki and ethical permission was granted from the North East London Research Committee (Ref. No. 08/H0702/52). Following obtaining fully informed consent, clinical, biochemical, and physiological data were collected. Data included tobacco and alcohol use, electrolytes and renal function, liver function tests, differential leukocyte counts and clotting parameters. Child-Pugh (CP) (29) and model for end-stage liver disease (MELD) (21) organ severity scores were calculated.

Sample Collection

Venous blood was collected from patients/volunteers into heparinised pyrogen-free tubes and was immediately pre-cooled to 4^{o}C until experimental use. Neutrophil granular phenotype and functional tests were performed within 1 hour of blood being drawn. Plasma was obtained by centrifuging whole blood at 4,500 rpm for 10 minutes at 4^{o}C and stored at -80^{o}C for subsequent cytokine determination by enzyme-linked immunosorbent assay (ELISA) and cytometric bead array (CBA).

Characterisation of neutrophil extracellular granular phenotype at baseline, and after stimulation with bacterial peptides

One hundred microlitre aliquots of heparinised blood were placed into pyrogen-free tubes containing 380 µL of Rosenwall Park Memorial Institute (RPMI)-1640 media (Sigma-Aldrich, U.K.). Samples were stimulated with either 20 µL of opsonised *E. coli* (1.5 x 10^{9} cells/ml) or 20 µL fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine) 0.2µM (Becton-Dickenson [BD} U.K.) along with control (phosphate-buffered saline [PBS]). Samples were incubated at 37^{o}C for 20 minutes and the reaction was stopped by adding 3 ml of PBS.

The incubated whole-blood cell pellet was then stained with fluorochrome-conjugated monoclonal antibodies [(anti-human CD66b IgM κ, anti-human CD63 IgG1 κ, anti-human CD11b IgG1 κ, anti-human myeloperoxidase (MPO) IgG1 κ, anti-human CD16 IgG1 κ) BD, UK] at room temperature in darkness for 30 minutes. After washing and centrifugation, red blood cells were lysed and re-suspended in 300 mL PBS and analysis using a FACS Canto II analyser and FACS Diva 6.1.2 software (BD, San Jose, CA). Granulocytes were gated from the

cell suspension based on forward and side scatter characteristics and the mean fluorescence intensity (MFI) of CD16 was measured. The CD16 positive granulocytes were gated as neutrophils and the MFI of CD11b, CD66b, CD63 and MPO was measured from this cell population.

Characterisation of neutrophil intracellular granular phenotype at baseline, and after stimulation with bacterial peptides

Samples were treated as previously described in the incubation and extracellular protocol, however for the intracellular staining, whole blood was first incubated with anti-CD16 antibody before lysis and fixation and then permeabilisation using a cytofix/cytoperm solution (BD, UK). Permeabilised cells were then labelled with fluorochrome-conjugated monoclonal antibodies (anti-human CD66b IgM κ, anti-human CD63 IgG1 κ, anti-human CD11b IgG1 κ, anti-human myeloperoxidase (MPO) IgG1 κ) before washing, re-suspension and FACS analysis.

Cytokine estimation

Plasma levels of the pro- and anti-inflammatory cytokines (IFN-γ, IL-10, IL-2, IL-6, IL-8 and TNF-α) were determined from plasma and supernatant samples previously stored at -80^{o}C using CBA (BD, UK) and results (pg/mL) were correlated with neutrophil granular phenotype, physiological and biological parameters.

Lactoferrin quantification

Baseline values of lactoferrin, representative of neutrophil secondary granular content were measured from patient plasma and incubation medium supernatants obtained by centrifugation at 4500rpm for 10 minutes at 4^{o}C and stored at -80^{o}C. Sandwich ELISA was performed using a Lactoferrin ELISA kit (Merck Millipore, Germany).

Image Stream Analysis

Data on the subcellular localisation of neutrophil granules was sought by using a similar incubation and fluorochrome-staining protocol for neutrophils from HC and ALD patients utilising an Image Stream^{x} Mark II Analyser (Amnis, Seattle, USA) which combines the phenotyping capability of flow cytometry with the detailed imagery and functional insights

of microscopy. Comparisons of MFI when gating at the intracellular and cell-surface compartments were made.

Statistical Analysis

Where appropriate, values are expressed as mean, median and inter-quartile range. A paired t-test was used for comparisons pre- and post-stimulation and Mann-Whitney U test was used for comparison between 2 groups. When comparing 3 or more groups simultaneously, the Kruskal-Wallis was utilised with Dunn's multiple comparison test. Pearson and Spearman correlations were used for parametric and nonparametric data, respectively. All statistical analyses were performed using GraphPad Prism 6.0 for Windows (GraphPad Software, Inc., San Diego, CA); $p<0.05$ was considered as statistically significant.

Results

Patient demographics and clinical parameters

The baseline demographics, biochemical parameters and disease severity scores of the patients studied are detailed in Table 1. There were trends towards slight increases in markers of liver disease severity and systemic inflammation seen in the actively drinking subset of ALD cirrhosis. In all patients there was no suspicion of active infection based upon clinical evaluation, biochemical and radiological findings.

In those with ALD cirrhosis, 4 (14%) patients developed bacterial infections over the course of 1 year of follow up with infections equally distributed across abstinent and actively drinking arms. One year mortality was measured at 14%, with 2 patients dying from complications of severe sepsis and MOF, 1 from severe hepatic decompensation and hepatorenal syndrome and 1 from unrelated causes. Three abstinent ALD cirrhotic patients were transplanted within 12 months of enrolment into this study.

Table 1

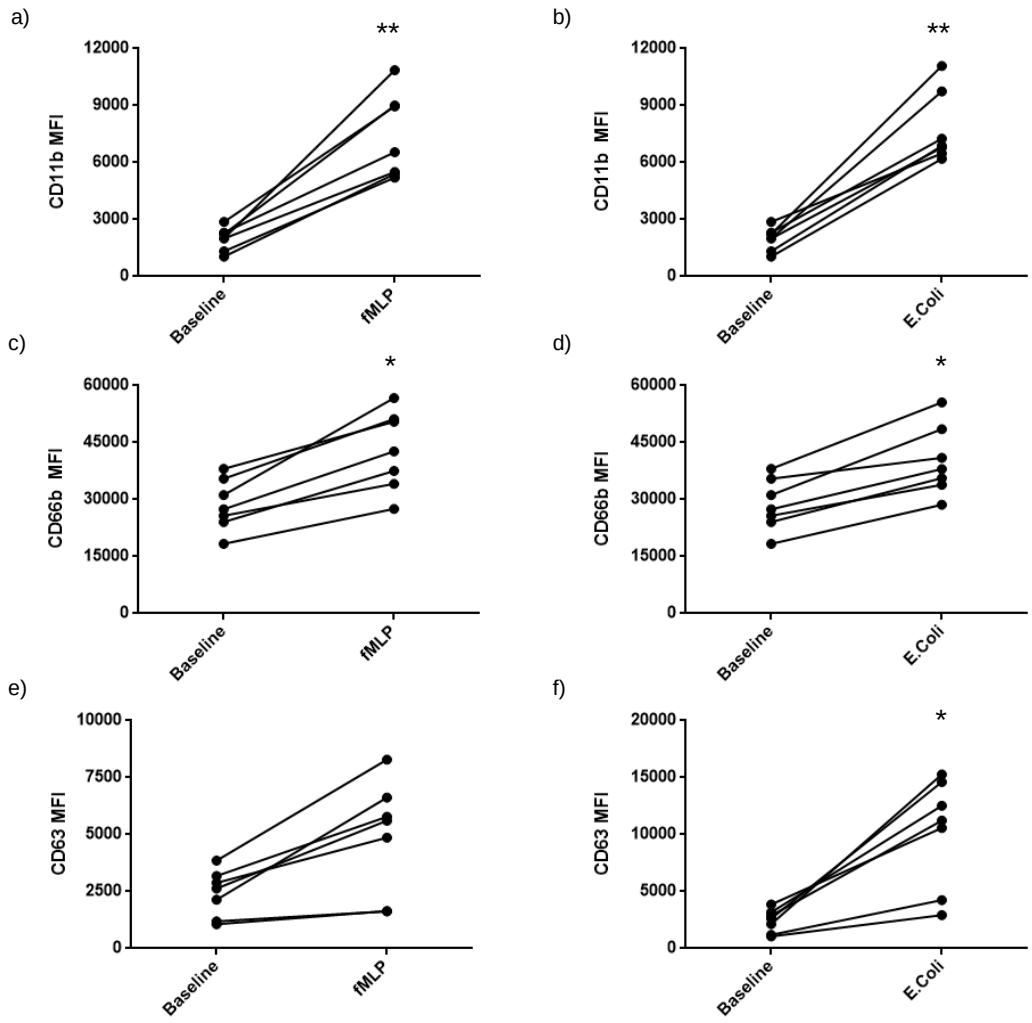
Study Group Data	Healthy Controls	Abstinent	Actively drinking	Total Patient Study Group
Number	12	15	14	29
Age	34 (31-54)	54 (45-59)	53 (47-59)	54 (47-59)
Sex Male	6	8	11	19
Female	6	7	3	10
Bilirubin (µmol/L)	-	40 (30-57)	123 (56-316)*	52 (32-141)
AST (iu/L)	-	35 (28-58)	93 (80-138)*	70 (39-94)
ALP (iu/L)	-	123 (93-178)	153 (133-258)	140 (106-192)
GGT (iu/L)	-	65 (37-109)	251 (111-503)*	109 (58-337)
Creatinine (µmol/L)	-	88 (62-103)	58 (43-90)	68 (52-99)
INR	-	1.53 (1.15-1.91)	1.56 (1.34-1.81)	1.53 (1.25-1.83)
Albumin (g/L)	-	32 (28-36)	27 (25-30)*	29 (26-35)
WCC ($x10^9$/L)	-	4.0 (3.4-5.6)	7.1 (5.7-10.6)*	5.64 (3.9-7.7)
Platelets ($x10^9$/L)	-	110 (67-129)	130 (110-181)	123 (92-148)
CRP (mg/L)	-	8.7 (5.3-17.7)	20.2 (11.0-42.1)*	12.1 (7.3-28.7)
Child Pugh Score	-	10 (7-11)	10 (9-12)	10 (8-11)
MELD Score	-	16 (10-21)	20 (16-25)*	18 (13-22)
UKELD Score	-	53 (52-58)	59 (55-62)*	56 (53-61)
Average Alcohol Intake (g/day)	-	0	143 (120-255)	-

Table 1: Study patient enrolment characteristics, describing age and sex distributions in addition to biochemical and organ severity scores between groups (expressed as median and inter-quartile range). MELD: model for end stage liver disease, UKELD: united kingdom model for end stage liver disease. Patients who were actively drinking had trends towards higher markers of liver disease and systemic inflammation (p<0.05).*

Neutrophil granular responses in healthy controls

Similar responses to stimulation in healthy controls were seen in both secretory vesicle and secondary granule mobilisation to the cell membrane when comparing stimulation with both fMLP and opsonised *E. Coli* [Figure 2 a + b, c + d]; however, primary granule responses were significantly elevated after stimulation with *E. Coli* ($p=0.0023$) compared to fMLP stimulation [Figure 2 e + f]. A proof of principle Image Stream Flow cytometry plot of healthy control neutrophil phenotype demonstrated significant upregulation of CD11b, CD66b and CD63 at the cell surface in response to stimulation with both fMLP and *E. Coli* in keeping with our FACS Canto flow cytometric data; this was not powered to detect differences between HC and ALD cirrhosis patients [Figure 3]. This is in keeping our understanding of granule physiology with respect to the different levels of calcium-dependant signalling required to mobilise specific granule subsets to the cell membrane [Figure 1] (3, 7).

Figure 2



***** **p < 0.05**

****** **p < 0.005**

Figure 2: Extracellular staining in healthy volunteers of fluorochrome-labelled neutrophil granule markers for secretory vesicles (CD11b) (a + b), secondary granules (CD66b) (c + d) and primary granules (CD63) (e + f) pre- and post-stimulation with fMLP and opsonised E. coli. Secretory vesicles and secondary granules were similarly significantly upregulated upon stimulation with fMLP, a potent neutrophil chemotaxin, or opsonised E. Coli, however primary granules showed a significantly increased response to bacterial stimulation over fMLP (p=0.002).

Figure 3

Figure 3: Image Stream Flow Cytometry plots of a healthy volunteer demonstrating fluorochrome-labelled staining patterns at the cell surface (a - c) and intracellularly (d - f), at baseline (a + d), after stimulation with the potent neutrophil chemotaxin fMLP (b + e), and after stimulation with opsonised E. Coli (c + f). Primary granules (column 1), secondary granules (column 5) and secretory vesicles (column 6) are all upregulated at the cell surface in response to fMLP and E. Coli stimulation with a more robust response observed to E.Coli. Plots from patients with ALD cirrhosis were indistinguishable and did not reveal impaired granule mobilisation or localisation.

Secretory Vesicles

CD11b, which is expressed in neutrophil secretory vesicles and on the cell surface and binds intercellular adhesion molecule 1 (ICAM-1) on the activated vascular endothelium to mediate neutrophil binding and initiate the process of extravasation, was significantly elevated in unstimulated neutrophils isolated from ALD patients when measured extracellularly (p=0.0001) [Figure 4a] and showed a tendency towards upregulation intracellularly. Secretory vesicle mobilisation to the cell membrane in response to bacterial stimulation was similar in ALD compared to HC, with no demonstrable deficit in secretory vesicular upregulation at the cell surface in response to bacterial stimulation in ALD cirrhosis.

Neutrophils examined from patients with ALD cirrhosis were activated at baseline, pre-primed to interact with and adhere to the vascular endothelium and had augmented responses to bacterial stimulation when compared to HC.

Secondary Granules

Secondary granule content and mobilisation, as measured by flow cytometric CD66b quantitation on extracellular staining, before and after bacterial stimulation, demonstrated no significant difference in the ALD cirrhosis cohort as compared against HC. Of note however, there was increased intracellular staining of secondary granules at baseline (p=0.025) with no impairment in extracellularisation of secondary granule content observed in the ALD cohort in keeping with a primed neutrophil phenotype. Baseline plasma levels of lactoferrin were significantly elevated [Figure 4b] in patients with ALD (p=0.035) compared to HC suggesting that neutrophils from patients with ALD cirrhosis display augmented secondary granule release in their circulating state.

Figure 4

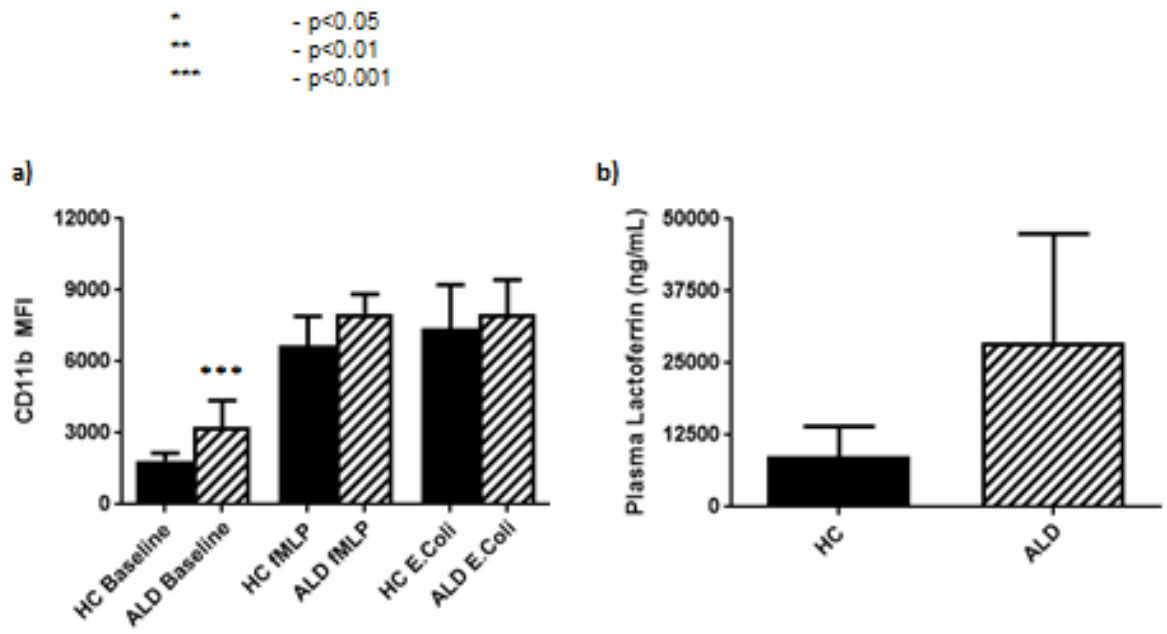


Figure 4: Cell-surface expression of CD11b (a) comparing patients with ALD cirrhosis against HC at baseline, and following stimulation with the neutrophil chemotaxin fMLP and opsonised E. Coli. Neutrophils in patients with ALD cirrhosis showed increased expression of CD11b at the cell surface at baseline (p=0.001) and no impairment of granular mobilisation to the cell surface after stimulation with bacterial peptides. There was neither a quantitative secondary granular deficit, nor an impairment of granule mobilisation observed in patients with ALD cirrhosis and plasma levels of lactoferrin (b) were increased at baseline when compared to healthy volunteers (p=0.035), suggestive of increased resting secondary granule release.

Primary Granules

CD63 expression was elevated when measured at the cell surface in patients with ALD cirrhosis both at baseline (p=0.0001) and after stimulation with fMLP (p=0.009) and *E. Coli* (p=0.0003) when compared against HC [Figure 5a]. This was matched by intracellular data which showed significant elevations in the intracellular concentrations of primary granules (p=0.0005) within the neutrophils of patients with ALD compared to HC [Figure 5b]. There was a tendency in ALD cirrhosis towards increased intracellular concentrations of MPO.

Abstinence versus active-alcohol consumption in alcohol-related cirrhosis

When compared to HC, ALD cirrhosis patients who were abstinent had significantly upregulated neutrophil granular function in comparison to HC (CD63 p<0.001, CD66b p<0.001); alcohol consumption appeared to abrogate the neutrophil pre-priming and exaggerated granular responses in ALD cirrhosis. Dampening of the exuberant neutrophil granular responses and apparent normalisation of their activated phenotype in active alcohol consumers were seen in all granule subsets examined [Figure 6] but were most significantly noted in the intracellular content of primary granules and secondary granules (p<0.0001).

Neutrophil granular phenotype and severity of liver disease

There was no observable difference in neutrophil granular phenotype categorised by MELD score; severity of liver disease did not appear to affect neutrophil granular function [Figure 7].

Plasma cytokine levels in alcohol-related cirrhosis

Plasma from patients with ALD cirrhosis had significantly upregulated levels of IL-8 in comparison to HC (p<0.001). This is in keeping with the observations of pre-primed neutrophil phenotype in a pro-inflammatory cytokine milieu; there were no significant differences observed in measurements of IFN-γ, IL-10, IL-2, IL-6, IL-8, TNF-α; however trends towards pro-inflammatory cytokine profiles were seen in ALD [Figure 8].

Figure 5

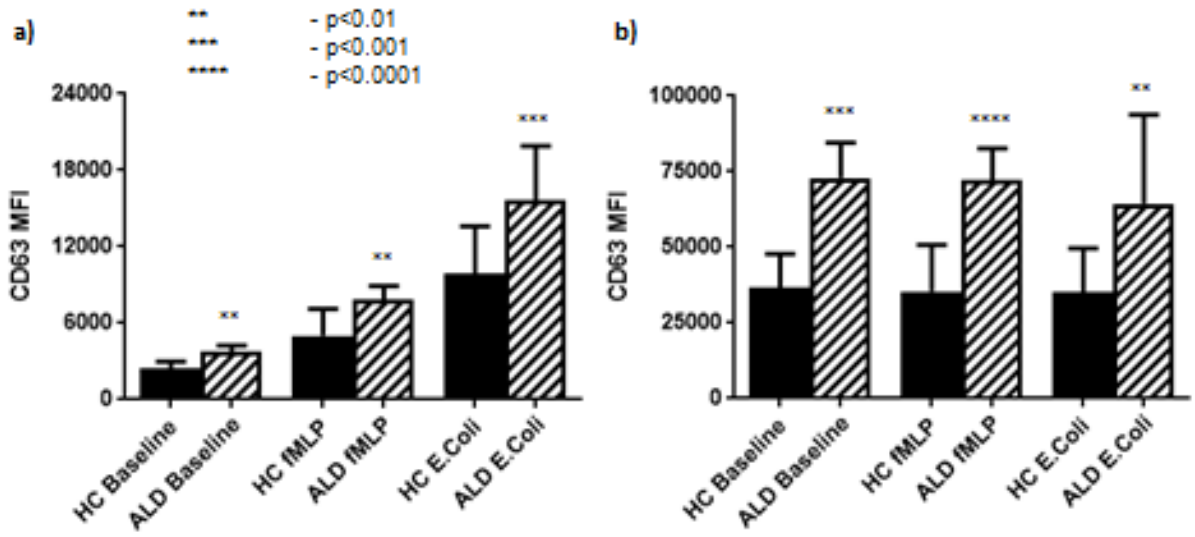


*Figure 5: Cell-surface (a) and intracellular (b) expression of CD63 comparing patients with ALD cirrhosis against healthy volunteers at baseline, and after stimulation with the neutrophil chemotaxin fMLP, and opsonised E. Coli. Neutrophils expressed significant upregulation at baseline in primary granule expression both intracellularly (p=0.0005: ***) and at the cell surface (p=0.001: **) in patients with ALD cirrhosis compared to healthy volunteers. Neutrophil primary granules were hyper-responsive with significant differences in mobilisation to the cell membrane after incubation with fMLP (p=0.009: **) and E. Coli (p=0.0003: ***).*

Figure 6

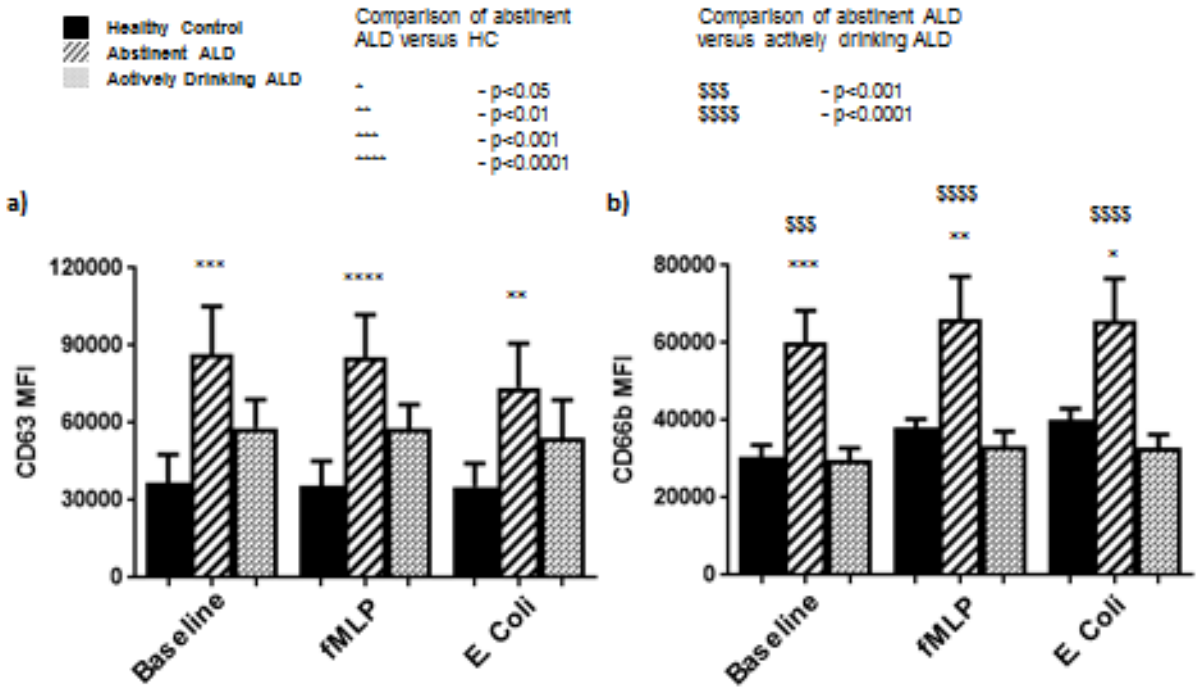


Figure 6: Intracellular expression of primary [a (CD63)] and secondary [b (CD66b)] granule markers comparing actively drinking patients with ALD cirrhosis against those who had been abstinent for >6 months and healthy controls (Kruskal-Wallis with Dunn's multiple comparison test analysis). Active alcohol consumption normalised the upregulated baseline granule expression and exaggerated response to bacterial stimulation that was seen across all granular subsets. The most significant impact was noted in intracellular staining of primary and secondary granules (p<0.001).

Figure 7

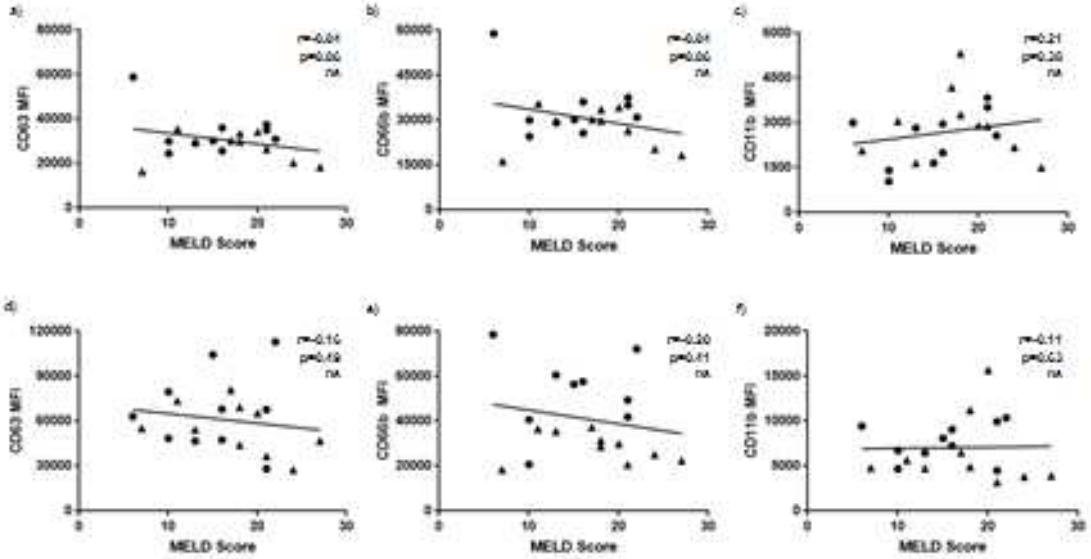


Figure 7: Correlation of extracellular (a, b, c) and intracellular (d, e, f) neutrophil granular marker expression at baseline in ALD cirrhosis when compared against MELD score. Severity of underlying liver disease did not correlate with changes in neutrophil granular expression at baseline or mobilisation in response to bacterial stimulus. Abstinent ▲ actively drinking ●

Figure 8

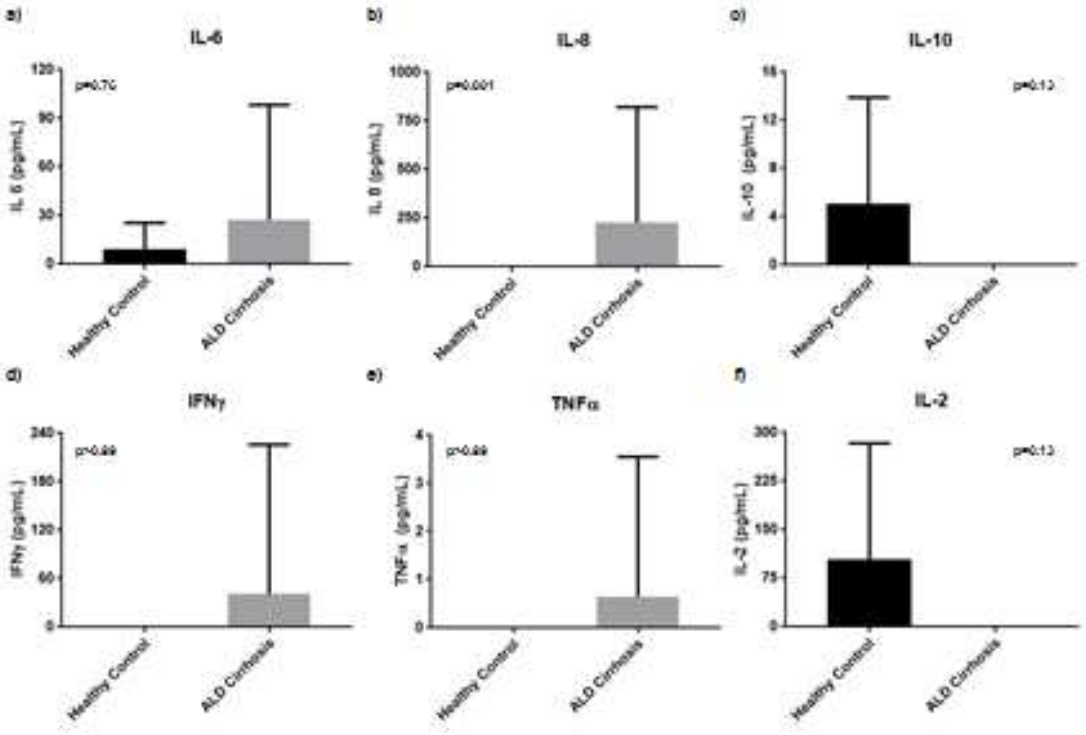


Figure 8: Supernatant cytokine measurements performed using cytokine bead array demonstrating non-significant trends towards upregulation of proinflammatory cytokines [a) IL-6, b) IL-8, d) INFγ and e) TNFα] in ALD cirrhosis. Conversely, anti-inflammatory cytokines [c) IL-10, f) IL-2] were downregulated in ALD cirrhosis.

Discussion

We have demonstrated, contrary to our *a priori* hypothesis, that neutrophil granular physiology in ALD cirrhosis is neither quantitatively deficient nor is there any demonstrable impairment in granule mobilisation and release. Neutrophil granule hypo-responsiveness is therefore unlikely to be contributing to the innate immune paresis observed in ALD cirrhosis. This finding is contradictory to other data with respect to ALD cirrhosis (9, 24); however this is the first time that individual neutrophil granular subsets have been described in this context. Other studies have shown separate defects in neutrophil cellular processes that go some way towards explaining this functional deficit in response to bacterial challenge, with impaired chemotaxis (12, 15, 20), opsonisation (26, 35) and phagocytosis (19, 30) reported.

Conversely, the pattern of neutrophil granule baseline expression confirms these neutrophils are activated in their circulating state in ALD cirrhosis. They have higher levels of CD11b both on their cell surface and intracellularly; by implication they are primed, more adhesive and liable to interact with the vascular endothelium. An elegant study published in 1983 reporting increased neutrophil adherence to nylon fibre *in vitro* in the context of ALD cirrhosis corroborates this data (2).

Furthermore, neutrophils from patients with ALD cirrhosis have increased staining of primary and secondary granules intracellularly at baseline and augmented granular release in response to chemotactic and bacterial stimuli when compared against HC. Neutrophils are therefore more likely to release their toxic granular contents in response to lower concentrations of bacterial stimulation and in higher levels than they are in healthy adults; this may lead to inappropriate and/or excessive granule release and tilt the spectrum of granule function towards creating an environment of oxidative stress and damage in host tissue. This paradigm of primed and inappropriately sensitised neutrophils gives weight to the suggestion that dysregulation of the innate immune system in ALD cirrhosis is a double-edged sword, both contributing to the propensity to develop bacterial and fungal sepsis, and, in terms of granule hyper-responsiveness, the propensity to suffer the serious sequelae of sepsis.

When comparing neutrophil granular phenotype in ALD cirrhosis patients who are actively drinking alcohol against those who are abstinent, intracellular content of both primary and secondary granules was significantly elevated in the abstinent cohort but these changes were not seen in patients who were actively drinking. Neutrophil activation and the production of ROS have been shown to lead to hepatic injury in this context (4) and selective gut decontamination can prevent hepatic injury related to alcohol toxicity (1). The effect of active alcohol consumption appears to dampen the neutrophil inflammatory responses across the board of neutrophil granular phenotype; this is in keeping with observations of the effects of alcohol on neutrophil ROS production (27, 34). Rebound hyper-responsiveness and increased neutrophil degranulation may cause bystander damage and worsen liver injury on cessation of alcohol consumption. This observation provides credence in the form of a proposed immunological mechanism for the frequently observed phenomenon of initial clinical deterioration on cessation of alcohol consumption however further mechanistic work is required here. We feel that the inclusion of actively drinking and abstinent ALD cirrhotic patients in our study is likely to explain the differences in observed granular phenotype described in recent papers from Markwick *et. al* and Boussif *et. al* (9, 24) whose patient cohorts were actively drinking patients with ALD cirrhosis and acute alcoholic hepatitis. Further studies are warranted to investigate the effects of active alcohol consumption upon neutrophil granule function, particularly interrogating whether this is a direct inhibitory effect on neutrophils or is related to a reduction in neutrophil priming via manipulation of the enteric microbiota and circulatory endotoxin burden.

Infection and its propensity to evolve into severe sepsis and MOF in the context of cirrhosis remains a huge challenge facing patients and clinicians alike. In the face of a tide of rising liver disease it is crucial that we understand the immunological aberrancies underpinning this propensity toward the development of sepsis and poor patient performance in controlling and fighting infection. This will allow the selective targeting of innate immunological pathways.

Figures and Tables

Table 1

Study patient enrolment characteristics, describing age and sex distributions in addition to biochemical and organ severity scores between groups (expressed as median and inter-quartile range). MELD: model for end stage liver disease, UKELD: united kingdom model for end stage liver disease. Patients who were actively drinking had trends towards higher markers of liver disease and systemic inflammation (* $p<0.05$).

Figure 1

Graphic representation of neutrophil function, demonstrating conversion from a circulating inactive immune cell to targeted degranulation and phagocytosis at sites of inflammation (modified from Borregaard *et. al* (7)). The process occurs via sequential control of neutrophil granules which contain specific arrays of proteins which serve specific and timely functions relating to neutrophil activation.

Initial interaction occurs with the vascular endothelium via selectins and cell adhesion molecules which facilitate rolling, adhesion and subsequently diapedesis across the endothelial membrane into the interstitial space. This in turn allows hierarchical activation of the neutrophil with mobilisation of granules to the cell surface and release of chemokines, cytokines and microbicidal proteins in a time ordered sequence, allowing the neutrophil to migrate to areas of inflammation and release microbicidal proteins and reactive oxygen species at targeted areas.

Figure 2

Extracellular staining in healthy volunteers of fluorochrome-labelled neutrophil granule markers for secretory vesicles (CD11b) (a + b), secondary granules (CD66b) (c + d) and primary granules (CD63) (e + f) pre- and post-stimulation with fMLP and opsonised *E. coli.* Secretory vesicles and secondary granules were similarly significantly upregulated upon stimulation with fMLP, a potent neutrophil chemotaxin, or opsonised *E. Coli*, however primary granules showed a significantly increased response to bacterial stimulation over fMLP ($p=0.002$).

Figure 3

Image Stream Flow Cytometry plots of a healthy volunteer demonstrating fluorochrome-labelled staining patterns at the cell surface (a - c) and intracellularly (d - f), at baseline (a + d), after stimulation with the potent neutrophil chemotaxin fMLP (b + e), and after stimulation with opsonised *E. Coli* (c + f). Primary granules (column 1), secondary granules (column 5) and secretory vesicles (column 6) are all upregulated at the cell surface in response to fMLP and *E. Coli* stimulation with a more robust response observed to *E.Coli*. Plots from patients with ALD cirrhosis were indistinguishable and did not reveal impaired granule mobilisation or localisation.

Figure 4

Cell-surface expression of CD11b (a) comparing patients with ALD cirrhosis against HC at baseline, and following stimulation with the neutrophil chemotaxin fMLP and opsonised *E. Coli*. Neutrophils in patients with ALD cirrhosis showed increased expression of CD11b at the cell surface at baseline (p=0.001) and no impairment of granular mobilisation to the cell surface after stimulation with bacterial peptides. There was neither a quantitative secondary granular deficit, nor an impairment of granule mobilisation observed in patients with ALD cirrhosis and plasma levels of lactoferrin (b) were increased at baseline when compared to healthy volunteers (p=0.035), suggestive of increased resting secondary granule release.

Figure 5

Cell-surface (a) and intracellular (b) expression of CD63 comparing patients with ALD cirrhosis against healthy volunteers at baseline, and after stimulation with the neutrophil chemotaxin fMLP, and opsonised *E. Coli*. Neutrophils expressed significant upregulation at baseline in primary granule expression both intracellularly (p=0.0005: ***) and at the cell surface (p=0.001: **) in patients with ALD cirrhosis compared to healthy volunteers. Neutrophil primary granules were hyper-responsive with significant differences in mobilisation to the cell membrane after incubation with fMLP (p=0.009: **) and *E. Coli* (p=0.0003: ***).

Figure 6

Intracellular expression of primary [a (CD63)] and secondary [b (CD66b)] granule markers comparing actively drinking patients with ALD cirrhosis against those who had been abstinent for >6 months and healthy controls (Kruskal-Wallis with Dunn's multiple comparison test analysis). Active alcohol consumption normalised the upregulated baseline granule expression and exaggerated response to bacterial stimulation that was seen across all granular subsets. The most significant impact was noted in intracellular staining of primary and secondary granules ($p<0.001$).

Figure 7:

Correlation of extracellular (a, b, c) and intracellular (d, e, f) neutrophil granular marker expression at baseline in ALD cirrhosis when compared against MELD score. Severity of underlying liver disease did not correlate with changes in neutrophil granular expression at baseline or mobilisation in response to bacterial stimulus. Abstinent▲, actively drinking ●.

Figure 8:

Supernatant cytokine measurements performed using cytokine bead array demonstrating non-significant trends towards upregulation of proinflammatory cytokines [a) IL-6, b) IL-8, d) INFγ and e) TNFα] in ALD cirrhosis. Conversely, anti-inflammatory cytokines [c) IL-10, f) IL-2] were downregulated in ALD cirrhosis.

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